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The Exon 8-Containing Prosaposin Gene Splice Variant Is Dispensable for Mouse Development, Lysosomal Function, and Secretion

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Prosaposin is a multifunctional protein with diverse functions. Intracellularly, prosaposin is a precursor of four sphingolipid activator proteins, saposins A to D, which are required for hydrolysis of sphingolipids by several lysosomal exohydrolases. Secreted prosaposin has been implicated as a neurotrophic, myelinotrophic, and myotrophic factor as well as a spermatogenic factor. It has also been implicated in fertilization. The human and the mouse prosaposin gene has a 9-bp exon (exon 8) that is alternatively spliced, resulting in an isoform with three extra amino acids, Gln-Asp-Gln, within the saposin B domain. Alternative splicing in the prosaposin gene is conserved from fish to humans, tissue specific, and regulated in the brain during development and nerve regeneration-degeneration processes. To elucidate the physiological role of alternative splicing, we have generated a mouse lacking exon 8 by homologous recombination. The exon 8 prosaposin mutant mice are healthy and fertile with no obvious phenotype. No changes were detected in prosaposin secretion or in accumulation and metabolism of gangliosides, sulfatides, neutral glycosphingolipids, neutral phospholipids, other neutral lipids, and ceramide. These data strongly indicate that the prosaposin variant containing the exon 8-encoded three amino acids is dispensable for normal mouse development and fertility as well as for prosaposin secretion and its lysosomal function, at least in the presence of the prosaposin variant missing the exon 8-encoded three amino acids.

Prosaposin is a multifunctional protein involved in a variety of biological processes. It is secreted or sorted to lysosomes. In lysosomes prosaposin is proteolytically processed to generate four sphingolipid activator proteins, known as saposins A to D, which are required for hydrolysis of sphingolipids by several lysosomal exohydrolases (52). Saposin A is the activator of galactocerebrosidase, which is the enzyme impaired in Krabbe disease, since a mouse model with a mutation in saposin A presents signs of late-onset Krabbe disease (37, 66). Saposin C deficiency leads to pronounced accumulation of glucosylceramide in the reticuloendothelial system and, consequently, results in the manifestation of Gaucher disease (47, 57). Saposin D was suggested to be involved in the hydrolysis of ceramide by ceramidase (28). A mouse model mutated in saposin D develops progressive polyuria and ataxia and ceramide accumulation in the kidney and the brain (36). Saposin B appears to be unique among the saposin family in that it stimulates enzymatic activity by interacting with the substrate rather than with the enzyme. It was suggested that saposin B acts as a detergent-like protein, solubilizing multiple lipid substrates for enzymatic hydrolysis (8). Mutations in saposin B lead to sulfatide accumulation and metachromatic leukodystrophy-like disease, which is similar to arylsulfatase A deficiency (23, 31, 48, 49, 63, 67). In saposin B deficiency there is also an excess of urinary excreting globotriaosylceramide, since saposin B is an in vivo activator of α-galactosidase A (51, 55).

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Secreted prosaposin is present in milk and cerebrospinal and seminal fluids (17, 27, 29). Many functions have been attributed to the secreted form, mainly in the nervous and the reproductive systems. It is a neurotrophic and myelinotrophic factor (18, 45), preventing apoptosis of neuronal cells in tissue culture (22, 60), and a neuroprotective and regenerative agent in vivo (30, 54).

Prosaposin is also an important factor in development, maintenance, and differentiation of male reproductive organs (40, 42). It is one of the major proteins secreted by Sertoli cells in the testes and is involved in spermatogenesis and fertilization (2, 13, 38, 59). In muscle tissue culture cells, prosaposin has been shown to have a myotrophic role in differentiation from myoblasts to myotubes (50).

Prosaposin was suggested to facilitate the transfer of glycosphingolipids between artificial membranes (21), and cell surface prosaposin may act as a sphingolipid binding protein. Since gangliosides and other glycosphingolipids exist as surface membrane constituents, prosaposin-ganglioside complexes at the cell surface may be involved in ganglioside function. However, the functional link between the neurotrophic and the glycosphingolipid transfer effects is unknown.

Deficiency in prosaposin is fatal. Two mutations in the prosaposin gene, leading to a total loss of prosaposin-saposins, were identified in patients (26, 56). In both cases an accumulation of multiple sphingolipids in the brain and visceral organs and a severe neurological disease including hypomyelination led to early death of the patients (15, 26, 56). Targeted disruption of the murine prosaposin gene resulted in a complex phenotype including severe central nervous system disease and

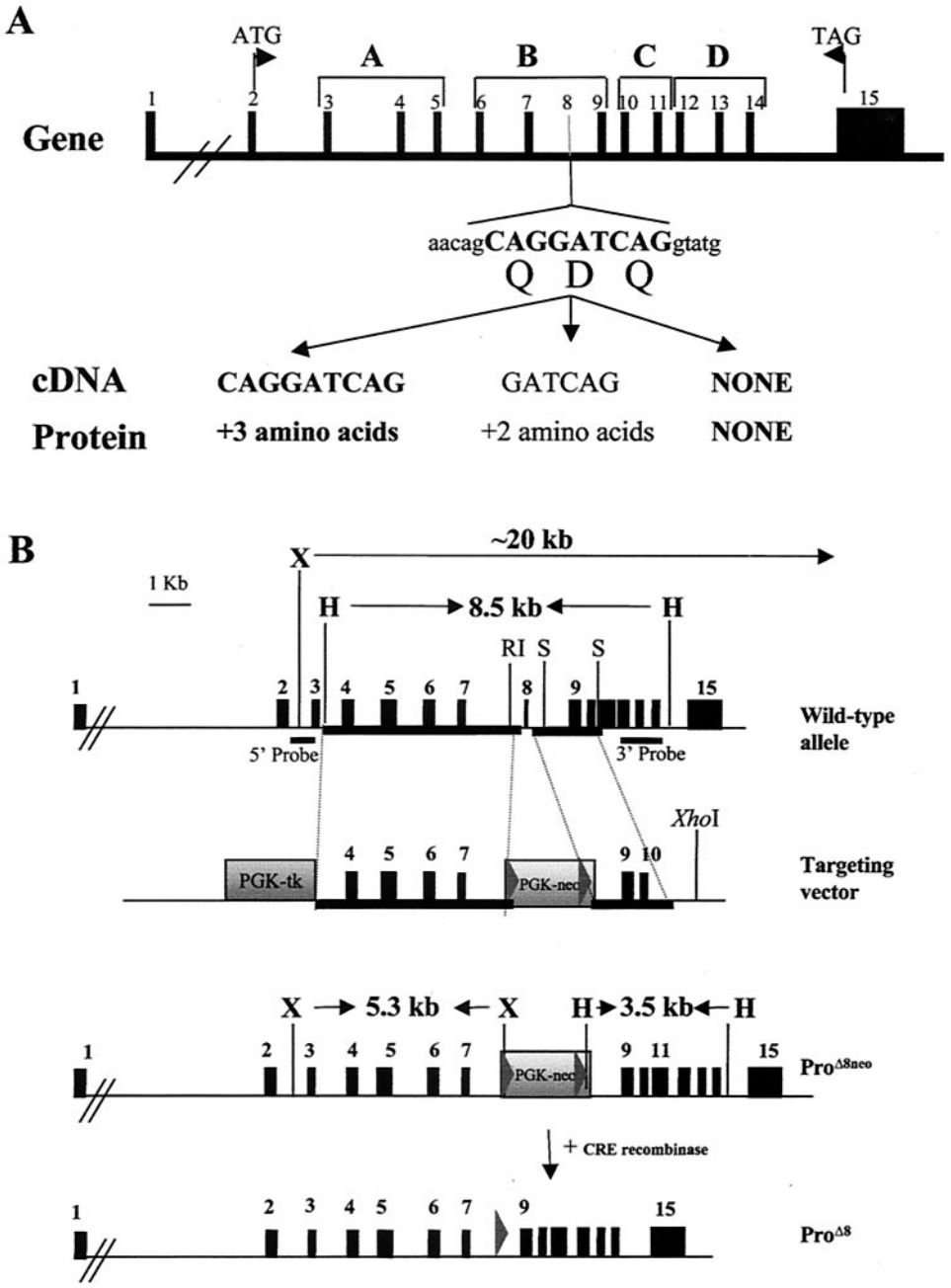

FIG. 1. Alternative splicing and deletion of mouse prosaposin exon 8. (A) Mammalian prosaposin gene with the alternatively spliced exon (exon 8) and the possible proteins that derive from it. The numbers above the boxes denote the exons. A, B, C, and D represent different saposins. (B) Strategy for exon 8 targeting. Partial restriction maps of the prosaposin gene, the targeting vector (pProΔ8neo^{KSloxpPNT}), and the targeted allele before (Pro$^{\Delta 8neo}$) and after (Pro$^{\Delta 8}$) the Cre recombination are shown. The targeting vector was designed to replace exon 8 with the PGK-neo cassette flanked by two *loxP* sites. The numbers above the boxes represent the prosaposin exons. The bold line represents the genomic fragments which were inserted within the targeting vector. The triangles represent the *loxP* sequences. H, HindIII; RI, EcoRI; S, SacI; PGK, mouse PGK promoter; tk, herpes simplex virus thymidine kinase; neo, neomycin resistance gene.

widespread storage of multiple sphingolipids. The mutant mice showed abnormalities similar to those of human patients with total prosaposin deficiency (11).

The prosaposin gene contains 15 exons. It is transcribed to several mRNAs, resulting from alternative splicing of the 9-bp exon 8 (23, 44) (Fig. 1A). Three prosaposin mRNAs, with 0, 6, or 9 bp of exon 8, have been detected in human, mouse, and rat tissues. Two prosaposin RNA species exist in chick tissues, with or without exon 8 (7). Expression of exon 8-containing mRNA is tissue specific in humans, rats, and mice (7, 19, 32, 68). Its level is high in brain, heart, and skeletal muscle and decreases after stab wound and ischemia in the rat brain (19). The level of exon 8-containing RNA rises during chicken and mouse brain development (7).

In mouse, rat, and human tissues exon 8 encodes the amino acids Gln-Asp-Gln, within the saposin B domain of prosaposin. Alternative splicing of the prosaposin gene was assumed to be the mechanism responsible for differential sorting of the different prosaposin forms and as a mechanism that adapts to variable sphingolipid composition of tissues by different saposin B isoforms (32, 35). The prosaposin containing the extra three amino acids was more efficiently secreted from HeLa cells infected with vaccinia virus-derived vectors and from stably transfected BHK cells than was the isoform that does not contain them (16, 35). The three-dimensional model of saposin B provided evidence that the site of insertion of the exon 8-derived three amino acids is within the loop between alpha helices 3 and 4 (1). Ham (12) proposed that the insertion of the additional residues in this site could lead to loss of capacity to bind lipids, therefore directing this prosaposin isoform for secretion. It was also shown that, when supplied in the medium, both human prosaposins, with and without the exon 8-encoded three amino acids, were endocytosed by cells, reached lysosomes, and directed there the activity of saposin B and saposin C (35).

Previous studies have indicated that alternative splicing of the prosaposin gene may change the lipid binding specificity of saposin B, presumably to adapt to the variable sphingolipid composition of tissues. Lamontagne and Potier (32) have shown that synthetic peptides derived from the saposin B domain (from Ser246 to Glu266), with or without the three-amino-acid insertion, have different binding affinities. Insertion of the Gln-Asp-Gln sequence completely abolished the capacity of the peptide to bind GM1-ganglioside, whereas its affinity for sulfatide and sphingomyelin was increased about fourfold and almost twofold, respectively (32). Another study showed that all saposin B forms, with and without the exon 8-encoded amino acids, can function as sulfatide-globotriaosylceramide activators when tested in loading studies with exogenously labeled lipids on prosaposin-deficient fibroblasts (16).

In order to investigate the biological role of the exon 8-containing isoform, we created a mouse mutant devoid of this 9-bp exon. Such mutant mice do not have any obvious phenotypic changes, strongly indicating that the exon 8-containing prosaposin variant is dispensable for normal mouse development and fertility as well as for prosaposin secretion and its lysosomal function.

MATERIALS AND METHODS

Tissue culture. TM3 (mouse Leydig cells) and TM4 (mouse Sertoli cells) were grown in Dulbecco's modified Eagle medium (DMEM) containing 5% fetal calf serum. All cells were grown at 37°C in the presence of 5% CO_2.

Preparation of MEFs. Embryos (13.5 days) were used to generate mouse embryonic fibroblasts (MEFs). Briefly, the head, limbs, and viscera were removed from the embryos, and carcasses were minced and then trypsinized in 0.05% trypsin without EDTA in phosphate-buffered saline (PBS) for 10 min at 37°C. Forty milliliters of medium was added for three to five embryos, and cells were centrifuged for 8 min at 1,000 × *g* at room temperature. Cells were collected and grown in DMEM containing 20% fetal calf serum.

Construction of the prosaposin gene targeting vector. A 4.5-kb HindIII-EcoRI fragment, spanning introns 3 to 7 (including exons 4 to 7) of the prosaposin gene, and a 2-kb Ecl136II (SacI) fragment, spanning introns 8 to 10 (including exons 9 and 10) of prosaposin, were isolated from a λ-FIX II mouse genomic clone (originating from a 129/SvEv genomic library; Stratagene, La Jolla, Calif.). HindIII-EcoRI and Ecl136II fragments were subcloned into pET-28 plasmids (Novagen, San Diego, Calif.) and were inserted as the 5′ and 3′ arms, respectively, in the loxP/PGK-neo/PGK-tk vector, in which the phosphoglycerate kinase-neomycin (PGK-neo) cassette is flanked by two loxP sites (14). The two arms were cloned on either side of the loxP/PGK-neo cassette, with the *neo* gene in the same transcriptional orientation as prosaposin. The 2-kb Ecl136II fragment was inserted into a Bsu15I blunt-ended site, and the 4.5-kb HindIII-EcoRI blunt-ended fragment was inserted into a blunt-ended BamHI site, yielding the targeting vector pProΔ8neo^{KSloxpPNT} (Fig. 1B), missing the last 360 bp of intron 7, exon 8, and the first 165 bp of intron 8.

Generation of mice lacking prosaposin exon 8. Gene-targeted 129/SvEv embryonic stem (ES) cells were produced by electroporating XhoI-linearized pProΔ8neo^{KSloxpPNT} into W4 (ES) cells (34), followed by selection with G418 and ganciclovir as described previously (64). Two hundred fifty double-resistant colonies were generated and examined by Southern blot analysis. Five properly targeted ES cell lines were identified, and three were used to produce chimeric males by morula aggregation. Chimeras were crossed with either 129/SvEv or Swiss Webster (SW) females to produce heterozygous Pro$^{\Delta 8neo}$ mice. In order to remove the PGK-neo cassette from the Pro$^{\Delta 8neo}$ allele, heterozygous Pro$^{\Delta 8neo}$ male mice were crossed with CMV-Cre transgenic females that exhibit high expression of Cre recombinase in the early embryo (62). The two independently targeted lines had the same phenotype; thus, only one line was further analyzed on the two backgrounds.

Genotyping of wild-type, Pro$^{\Delta 8neo}$, and Pro$^{\Delta 8}$ alleles. Genomic DNA from either ES cells, mouse tails, or livers was digested with XmaI or HindIII, electrophoresed through an 0.8% agarose gel, and transferred to a Hybond N^+ membrane (Amersham/Pharmacia Biotech, Piscataway, N.J.). Hybridization was performed with ^{32}P-labeled DNA probes. A 5′ external probe was amplified from genomic DNA with the primers 5′-GATGGTGATGAATGAACATG-3′ and 5′-CCCAGCTTCGGTGACAAC-3′ to yield a 480-bp fragment spanning prosaposin intron 2 and exon 3. A 3′ external probe was amplified from a mouse prosaposin cDNA (58) with the primers 5′-GGTTTGAGCAACGCATGGC G-3′ and 5′-CCATGTTCTGACACCAGTAGC-3′ to yield a 570-bp fragment spanning exons 11 to 14.

The 5′ side of the recombinant Pro$^{\Delta 8neo}$ allele was examined with XmaI digestion and probed with the 5′ probe, which detected a ~20-kb wild-type fragment and a 5.3-kb fragment in the Pro$^{\Delta 8neo}$ allele (data not shown). The 3′ side was analyzed by HindIII digestion and probed with the 3′ probe, which detected an 8.5-kb wild-type fragment and a 3.5-kb mutant fragment (Fig. 2A).

For PCR analysis three primers were used: 5′-CCACAGTACGGATAGAA C-3′ (primer 1), 5′-GGTAGCTACACCGTCTGG-3′ (primer 2), and 5′-GCCA CTTGTGTAGGCGCCAAG-3′ (primer 3). Primers 1 and 2 (located within introns 7 and 8, respectively) yielded a 724-bp fragment from the wild-type allele and a 308-bp fragment from the Pro$^{\Delta 8}$ recombinant allele, missing the *neo* gene. Primers 1 and 3 (located within intron 7 and the PGK-neo cassette, respectively) amplified a 299-bp fragment of the Pro$^{\Delta 8neo}$ allele (Fig. 2B).

Detection of mouse prosaposin RNA by RT-PCR. Total RNA was extracted using TRI reagent (Molecular Research Center, Inc., Cincinnati, Ohio). Two to five micrograms was reverse transcribed in 25 μl, with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, Wis.). The resulting cDNA was used to amplify a 587-bp fragment spanning the exon 6 to 11 region of mouse prosaposin with the primers 5′-GCCAGGACTGTATGAAG-3′ and 5′-GGCA GCACAGAGGCCGAT-3′, as sense and antisense, respectively. Thermal cycling consisted of 94°C for 10 min, followed by 30 cycles of denaturation (94°C, 1 min), annealing (56°C, 1 min), and extension (72°C, 1 min) and a final extension at 72°C for 10 min. In order to differentiate between the mRNAs containing or missing exon 8, the reverse transcription-PCR (RT-PCR) products were digested with the restriction enzyme AlwI.

For quantification of the relative abundance of mouse prosaposin isoforms, the sense primer was labeled with the fluorescent dye 6-FAM (Integrated DNA Technologies, Inc., Coralville, Iowa). PCR amplification and AlwI cleavage were performed as described above. A volume of 1 to 2 μl was loaded onto a 5% Long Ranger (Biowhittaker Molecular Applications, Rockland, Maine) acrylamide–6 M urea gel and submitted to electrophoresis with an ABI 377XL DNA sequencer. Genescan Analysis 3.1 software (Applied Biosystems, Foster City, Calif.) was used to determine the size and the amount of the digested RT-PCR products. TAMARA 500 (Applied Biosystems) was used as an internal standard (for an example see Fig. 4B).

Protein analysis. Cells and mouse tissues were homogenized in lysis buffer (10 mM HEPES, 100 mM NaCl, 1 mM $MgCl_2$, 1% Triton X-100, and protease inhibitor mix [1 mM phenylmethylsulfonyl fluoride, 1 μg of aprotinin/ml, and 1 μg of leupeptin/ml {Sigma, St. Louis, Mo.}]). Homogenates were centrifuged at 10,000 × *g* for 15 min at 4°C, and supernatant protein levels were determined by the Bradford assay (6). Proteins were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) and were electroblotted

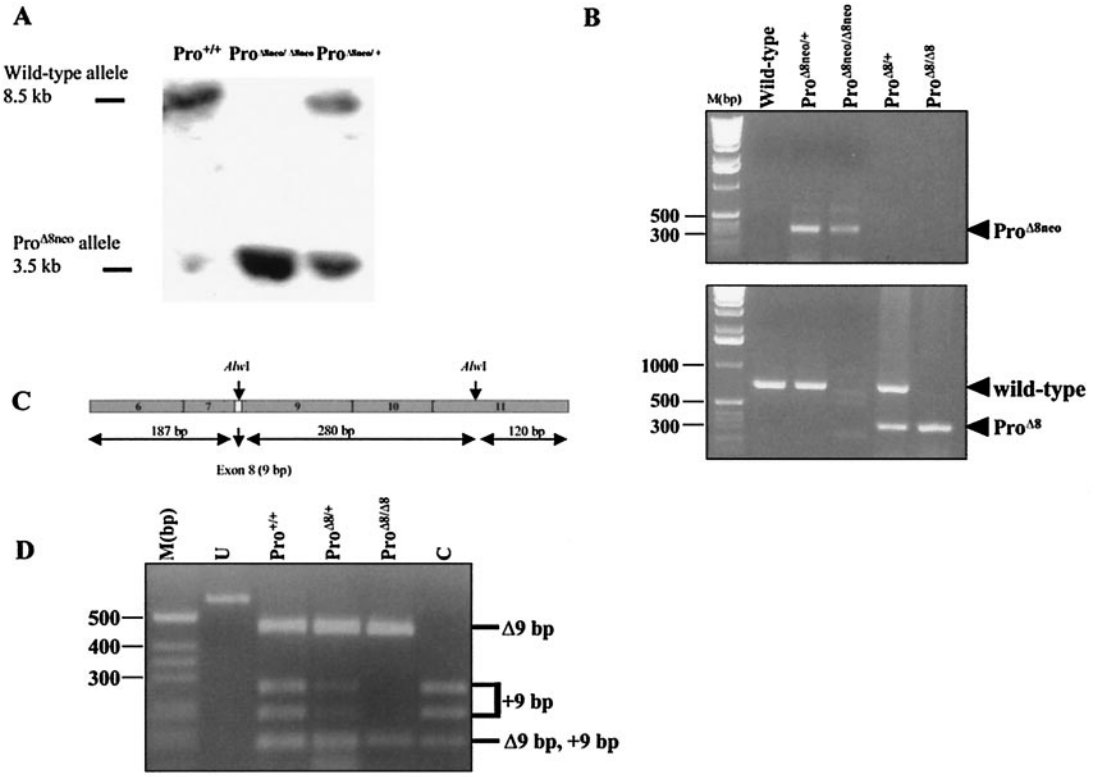

FIG. 2. Characterization of the prosaposin mutant mice. (A) Southern blot analysis of DNA from mouse liver, which was digested with HindIII. The 3′ probe hybridized with an 8.5-kb fragment in the wild-type allele and a 3.5-kb fragment in the $Pro^{\Delta 8neo}$ allele. Shown are wild-type ($Pro^{+/+}$), heterozygous ($Pro^{\Delta 8neo/+}$), and homozygous ($Pro^{\Delta 8neo/\Delta 8neo}$) genotypes. (B) PCR analysis of tail genomic DNA. A 724-bp fragment in the wild-type allele and a 308-bp fragment in the $Pro^{\Delta 8}$ allele were amplified using primers within introns 7 and 8 (lower panel). For amplification of the $Pro^{\Delta 8neo}$ allele, primers located within intron 7 and the PGK-neo cassette were chosen, yielding a 299-bp fragment (upper panel). (C) Schematic representation of the prosaposin cDNA. There is an additional AlwI restriction site within exon 8. The numbers within the boxes denote the exons. (D) RT-PCR analysis of brain prosaposin mRNA from different mice. An ethidium bromide-stained 1.5% agarose gel of RT-PCR products after digestion with AlwI is shown. The exon 8-containing isoform with the 9-bp sequence yielded three fragments, 280, 187, and 120 bp in size, while the isoform without exon 8 yielded two fragments, 458 and 120 bp in size. U, uncut PCR product; C, PCR product of a plasmid harboring exon 8-containig prosaposin DNA.

onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). After 1 h of blocking with PBS containing 5% skim milk, blots were incubated with anti-mouse prosaposin antisera (7). The blots were washed three times with PBS–0.1% Tween 20. After incubation with the secondary antibody (peroxidase-conjugated goat anti-rabbit immunoglobulin G; Jackson ImmunoResearch, West Grove, Pa.) and subsequent washing, prosaposin was detected using the Western blotting Luminol reagent (Santa Cruz Biotechnology, Santa Cruz, Calif.).

Immunohistochemical staining. Mouse organs were fixed in Bouin's fixative (saturated picric acid, 40% formaldehyde, and glacial acetic acid [75:25:5, vol/vol/vol]) and embedded in paraffin. Six-micrometer microtome sections were prepared and fixed on 2% Tespa-pretreated slides (Sigma). Prosaposin was detected using polyclonal anti-recombinant mouse prosaposin antibodies. The antibodies were produced against mouse prosaposin expressed in bacteria, as described elsewhere (7).

Metabolic labeling and immunoprecipitation. Cells were incubated for 30 min in Met-Cys-free DMEM at 37°C and then incubated in medium containing 100 μCi of [^{35}S]Met-Cys for 1 h at 37°C. At the end of labeling and after 2 h of chase the medium was collected and the cells were washed and lysed as described for protein analysis. Total radioactivity was measured, and equal amounts of counts per minute were precleared with 10 μl of protein A beads (Roche Diagnostics, Mannheim, Germany), in 450 μl of PBS containing protease inhibitors. The samples were rotated for 1 h at room temperature, followed by a short centrifugation. Supernatants were immunoprecipitated with 10 μl of anti-mouse prosaposin antisera and 20 μl of protein A beads at 4°C overnight. Samples were washed three times with 500 μl of PBS containing protease inhibitors, resuspended in 2× sample buffer, boiled for 5 min, centrifuged briefly, and resolved through SDS–5 to 20% PAGE. The gels were electroblotted onto a nitrocellulose membrane (Schleicher & Schuell), and radioactive signal was detected using exposure to X-ray films.

To immunoprecipitate prosaposin from serum, blood was centrifuged for 5 min at 5,000 × *g* at room temperature and serum was collected. Samples containing 800 μg of protein were immunoprecipitated as described above and resolved by SDS–10% PAGE. Protein detection was performed as described under "Protein analysis."

Lipid analysis. Lipids were extracted from organs as described previously (4). Briefly, tissues were homogenized in 40 ml of chloroform-methanol (2:1, vol/vol) and lipids were extracted for 4 h at room temperature. Protein levels were determined on aliquots of the homogenate (6). Tissue debris was removed by filtration of the homogenate with ashless paper (Whatman International, Maidstone, England). The homogenate was partitioned into a lower phase and an upper phase after addition of 10 ml of deionized water (9). Gangliosides and sulfatides were purified from the upper phase with a DEAE Sephadex column as previously described (5, 61). The lipids (corresponding to equal amounts of homogenate protein) were spotted on silica gel 60 thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) along with authentic standards and separated with chloroform–methanol–0.2% $CaCl_2$ (55:45:10, vol/vol/vol) as the developing solvent. The gangliosides were visualized using recorsinol followed by charring of the plate to detect sulfatides.

Neutral glycosphingolipids, ceramides, phospholipids, and neutral lipids were purified from the Folch lower phase by aminopropyl solid-phase extraction (LC-NH_2; Supelco, Bellefonte, Pa.) (4). Neutral glycosphingolipids were separated by TLC with chloroform-methanol-deionized water (65:25:4, vol/vol/vol) as the developing solvent and visualized with orcinol. Ceramides were separated using chloroform-methanol-acetic acid (190:9:1, vol/vol/vol). Phospholipids were

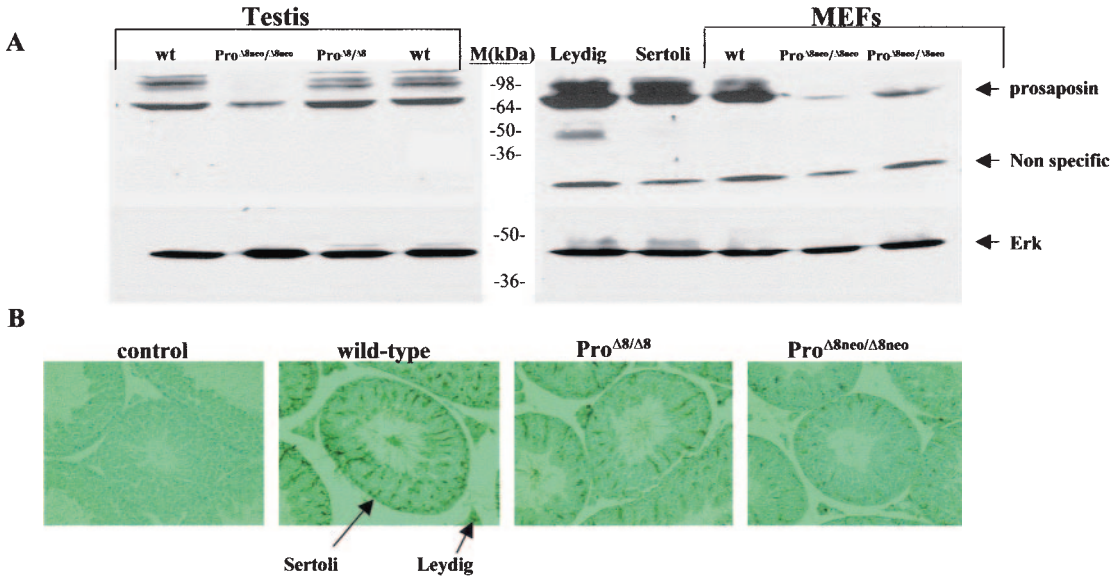

FIG. 3. Prosaposin expression in wild-type and exon 8-deletion mice. (A) Western analysis of testes and cultured cells. Testis extracts from wild-type, $Pro^{\Delta 8neo/\Delta 8neo}$, and $Pro^{\Delta 8/\Delta 8}$ mice and lysates of Leydig (TM3) and Sertoli (TM4) cells and MEFs that derived from wild-type or $Pro^{\Delta 8neo/\Delta 8neo}$ mice were prepared. Samples were electrophoresed through SDS-polyacrylamide gels (10 to 20% polyacrylamide), and the corresponding immunoblot was reacted with anti-mouse prosaposin antibodies. Detection was performed with horseradish peroxidase-conjugated goat anti-rabbit antibodies. Membranes were reblotted with anti-Erk antibodies to determine total protein levels. (B) Immunohistochemical analysis of testes. Sections of testes of adult mice were fixed in Bouin's fixative and embedded in paraffin. Slides were reacted with rabbit anti-mouse prosaposin antibodies. Detection was performed with horseradish peroxidase-conjugated goat anti-rabbit antibodies in the presence of horseradish peroxidase substrate (3′,3′-diaminobenzodine in 0.3% H_2O_2). All sections were then lightly counterstained with hematoxylin to visualize cells. Control, nonimmune serum; $Pro^{\Delta 8/\Delta 8}$, mice in which the PGK-neo cassette was removed by Cre recombination; $Pro^{\Delta 8neo/\Delta 8neo}$, mice in which the mutant allele contains the PGK-neo cassette.

resolved using chloroform-acetone-methanol-acetic acid-deionized water (50:20:10:15:5, vol/vol/vol/vol/vol) as the solvent system. Neutral lipids were separated using hexane-diethyl ether-acetic acid (80:20:1, vol/vol/vol) that was used on the full length of the TLC plate, followed by petroleum ether-diethyl ether-acetic acid (40:60:0.1, vol/vol/vol) on one-third of the plate. Ceramides, phospholipids, and neutral lipids were visualized using copper sulfate reagent and heating of the TLC plates at 180°C for 10 min. A neutral glycosphingolipid standard mixture, a monosialo- and disialoglycosphingolipid standard mixture, a neutral lipid mixture, a neutral phospholipid mixture, ceramides, and sulfatides were from Matreya (Pleasant Gap, Pa.).

RESULTS

Creating mice lacking exon 8 of prosaposin. To define the function of the mouse exon 8-containing prosaposin isoform, we deleted exon 8, with the last 360 bp of intron 7 and the first 165 bp of intron 8. Two heterozygous mutant lines lacking exon 8 were established, using gene targeting in ES cells (see Materials and Methods), one with the PGK-neo cassette within the prosaposin gene ($Pro^{\Delta 8neo/+}$) and one without it ($Pro^{\Delta 8/+}$) (Fig. 1B). From each line heterozygous mice were intercrossed, and the ratio of the three genotypes was found to be within the expected Mendelian range. Homozygous mutant mice ($Pro^{\Delta 8neo/\Delta 8neo}$, $Pro^{\Delta 8/\Delta 8}$) at all ages appeared healthy and indistinguishable from wild type. Histological analysis of different tissues and weight measurements at 6, 13, and 18 weeks did not show any difference between mutant and wild-type mice (data not shown).

To verify that the gene targeting event eliminated the production of the exon 8-containing prosaposin mRNA isoform, RT-PCR analysis was performed on RNA derived from brains of wild-type and homozygous mutant mice ($Pro^{\Delta 8/\Delta 8}$). To differentiate between prosaposin mRNAs with or without exon 8, the RT-PCR products were digested with the restriction enzyme AlwI, which has a cleavage site within exon 8 (Fig. 2C). The results clearly showed that, whereas wild-type and heterozygous mice had the two prosaposin isoforms, homozygous mutant mice expressed only the prosaposin isoform devoid of exon 8 (Fig. 2D). It is worth mentioning that to date there is no way to differentiate between the different prosaposin isoforms at the protein level, since available antibodies recognize both isoforms.

Prosaposin expression differs in the two mutant mice. To determine the expression levels of prosaposin in the mutant mice, we performed Western blotting and immunohistochemical analysis. As shown in Fig. 3A, Western blot analysis of testis revealed several prosaposin isoforms: one of 65 kDa, two isoforms higher than 70 kDa, and one of 98 kDa in size. The different forms may correspond to different glycosylation states of prosaposin or to a prosaposin-lipid complex (10, 21, 39). Lower
prosaposin levels were obvious in the testis of the $Pro^{\Delta 8neo}$ homozygotes, while the $Pro^{\Delta 8}$ homozygotes had levels comparable to that of wild-type mice. This result indicates that the insertion of the PGK-neo cassette within the prosaposin gene reduced the level of prosaposin and the removal of the PGK-neo cassette by Cre recombinase restored a normal expression level. This result was confirmed by immunohistochemical analysis of testis of the different mice (Fig. 3B). In testis from $Pro^{\Delta 8neo/\Delta\ 8neo}$ mice a weaker prosaposin staining of Leydig

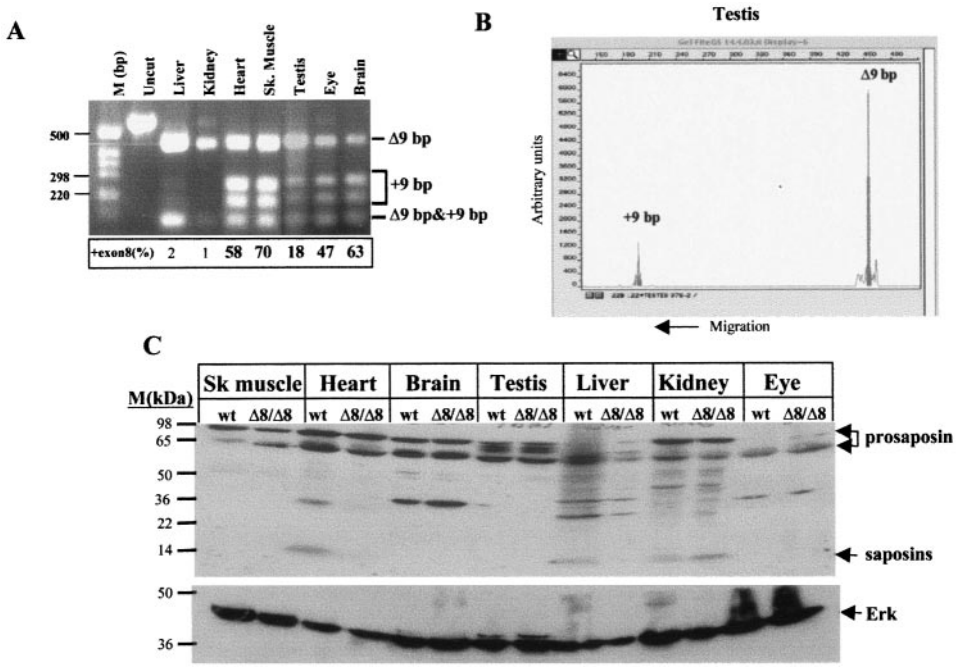

FIG. 4. Prosaposin expression in different tissues. (A) RNA was extracted from different tissues. The corresponding prosaposin RT-PCR products were electrophoresed through a 1.5% agarose gel, following digestion with AlwI. After digestion with AlwI, the exon 8-containing isoform, with 9 bp, yielded three fragments, 280, 187, and 120 bp in size, while the isoform without exon 8 yielded two fragments, 458 and 120 bp in size. (B) A representative scan of electrophoresis through a denaturing sequencing gel is shown. Since only the sense primer was fluorescently labeled, the 187-bp fragment represented the exon 8-containing isoform, while the 458-bp fragment represented the isoform lacking it. (C) Samples of different tissue extracts were electrophoresed through SDS–10 to 20% polyacrylamide gels, and the immunoblot was reacted with rabbit anti-mouse prosaposin antibodies. Detection was performed with horseradish peroxidase-conjugated goat anti-rabbit antibodies. The membrane was reblotted with anti-Erk antibodies to determine total protein levels. The amounts loaded on the gel (in micrograms) were as follows: skeletal muscle, 200; heart, 300; brain, 150; testis, 200; liver, 200; kidney, 350; and eye, 65. wt, wild type; Δ8/Δ8, mice in which the PGK-neo cassette was removed by Cre recombination. The percentages of exon 8-containing prosaposin RNA in the different samples are as follows: liver, 2; kidney, 1; heart, 58; skeletal muscle, 70; testis, 18; eye, 47; brain, 63.

cells (filling the interstitium between the tubules), of Sertoli cells, and in the lumen of seminiferous tubules was detected compared to the wild-type and the $Pro^{\Delta 8/\Delta 8}$ mice. To further delineate the differences in prosaposin level, MEFs were prepared from wild-type mice and from two different lines of $Pro^{\Delta 8neo/\Delta\ 8neo}$ mice. As shown in Fig. 3A, MEFs from $Pro^{\Delta 8neo}$ homozygotes presented lower prosaposin levels (~50%, as determined by densitometric analysis) than did wild-type MEFs or Sertoli (TM4) or Leydig (TM3) cells. Notably, in tissue culture cells only two prosaposin isoforms, of 65 and ~70 kDa, were observed.

We next examined the tissue expression of the exon 8-containing prosaposin RNA isoform. As shown in Fig. 4A, this isoform is abundant in brain, heart, and skeletal muscle; less so in testis and almost absent in visceral organs. To test whether exon 8 deletion alters the prosaposin expression pattern in tissues that normally express exon 8, Western blot analysis was performed on protein extracts derived from different organs isolated from wild-type or from the $Pro^{\Delta 8/\Delta 8}$ mice. As shown in Fig. 4C, two major prosaposin isoforms were detected in most tissues, 65 and 98 kDa in size, whereas in testis four different protein isoforms were expressed. Other prosaposin isoforms, smaller than 65 kDa, were obvious as well. They may correspond to intermediates in prosaposin processing to saposins (20, 33). In all tested tissues the prosaposin levels in the $Pro^{\Delta 8/\Delta 8}$ mice were comparable to those of wild-type mice. The results strongly indicate that in $Pro^{\Delta 8/\Delta 8}$ mice the absence of the exon 8-encoded three amino acids did not affect the expression level or the pattern of prosaposin products in tissues that normally express exon 8, such as skeletal muscle, heart, brain, eye, and testis. Instead, the specific removal of exon 8 by homologous recombination resulted in the replacement of the exon 8-containing isoform by the isoform lacking it in tissues that normally express exon 8.

Prosaposin without the three-amino-acid insertion is secreted. We further tested whether mutant mice lacking exon 8 secrete prosaposin. Detection of secreted prosaposin was performed by immunoprecipitation with anti-mouse prosaposin antibodies, of serum taken from wild-type, $Pro^{\Delta 8neo/\Delta 8neo}$, and $Pro^{\Delta 8/\Delta 8}$ mice, followed by Western blot analysis. The level of secreted prosaposin in serum from mutant mice was not obviously different from that of wild-type mice when normalized according to albumin levels (Fig. 5A). Prosaposin secretion from tissue culture cells was also examined. To this end, MEFs derived from normal or $Pro^{\Delta 8neo/\Delta 8neo}$ mice, as well as TM3 and TM4 cells, were metabolically labeled for 1 h with [^{35}S]Met-Cys and prosaposin was immunoprecipitated from the culture medium after 2 h of chase. The results presented in Fig. 5B show that, after 2 h of chase, prosaposin was detectable in the medium of all tested cells.

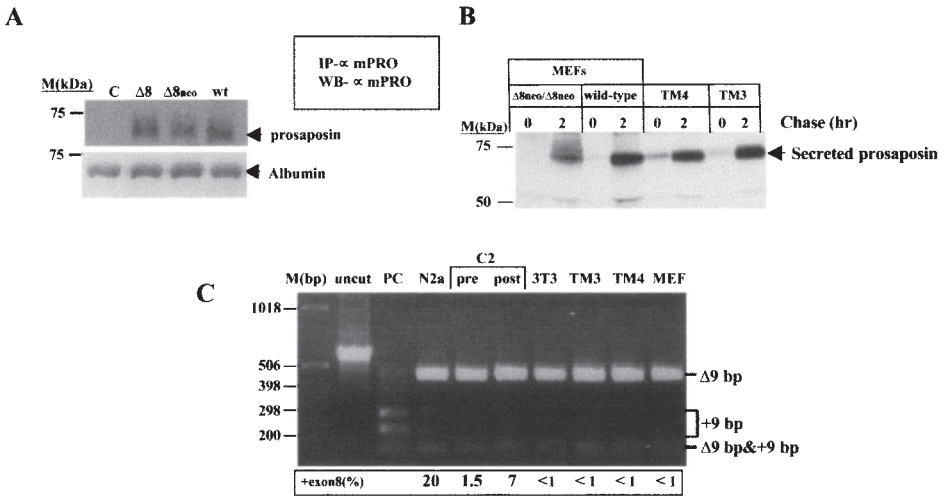

FIG. 5. Prosaposin secretion in wild-type and exon 8-deletion mice. (A) Eight hundred micrograms of mice serum was immunoprecipitated with anti-mouse prosaposin antibodies and electrophoresed through an SDS–10% polyacrylamide gel. The immunoblot was reacted with rabbit anti-mouse prosaposin antibodies, and detection was performed with horseradish peroxidase-conjugated goat anti-rabbit antibodies. Samples of the total serum were electrophoresed in parallel and were followed by Coomassie blue staining. Serum albumin was visualized for quantification. C, immunoprecipitation with nonimmunized rabbit serum; IP, immunoprecipitation; WB, Western blotting. The percentages of secreted prosaposin, compared to wild type (100%), are as follows: $Pro^{\Delta 8/\Delta 8}$ (Δ8), 127; $Pro^{\Delta 8neo/\Delta 8neo}$ (Δ8neo), 89. (B) Wild-type or $Pro^{\Delta 8neo/\Delta 8neo}$ MEFs and TM3 and TM4 cells were pulsed with [^{35}S]Cys-Met for 1 h. Following 2 h of chase, medium was collected and immunoprecipitated with a rabbit anti-mouse prosaposin antiserum. The immunocomplexes were separated by SDS–5 to 20% PAGE and visualized by autoradiography. (C) RT-PCR analysis of prosaposin mRNA from different mouse cultured cells. Shown is an ethidium bromide-stained agarose gel of RT-PCR products after digestion with AlwI. N2a, C2 cells before and after differentiation; NIH 3T3 and TM3, Leydig cells; TM4, Sertoli cells; PC (positive control), a plasmid harboring prosaposin cDNA containing exon 8. The 280- and 187-bp fragments indicate the presence of exon 8 (Fig. 2C). The percentage of exon 8-containing isoform was calculated using Genescan. The data represent the means of at least three separate measurements.

When the abundance of the different prosaposin RNA forms was tested in different mouse tissue culture cells, we noted no or very low expression of the exon 8-containing prosaposin isoform. We extended this study to include neuroblastoma (N2a) cells, muscle cells before and after differentiation (65), and NIH 3T3 cells. In all tissue culture cells there was no or low-level expression of the exon 8-containing prosaposin RNA (Fig. 5C). In contrast, in human tissue culture cells, the level of exon 8-containing RNA was consistently high (data not shown). The results clearly demonstrate that prosaposin is secreted in the absence of the exon 8-encoded three amino acids (due to their deletion or lack of expression) and show that expression of the exon 8-containing RNA is lost or reduced in cultured mouse but not human cell lines compared to in vivo tissues.

Lack of exon 8 and reduced prosaposin levels do not affect fertility. The biological roles attributed to the secreted prosaposin form and its localization in many secreting cells in male and female reproductive systems (13, 38, 41, 58, 59) prompted us to test the fertility of mice carrying the two mutant alleles. No differences in the average number of offspring per litter between the $Pro^{\Delta 8neo/\Delta 8neo}$ (8.83 ± 3.04) and the wild-type (10.42 ± 2.89) mice could be observed. Immunohistochemical staining of male and female reproductive organs showed similar results (data not shown). Based on the results, no function for the exon 8-encoded three amino acids in the reproductive system is observed.

Absence of prosaposin exon 8 does not change lipid metabolism. Previous work suggested that alternative splicing of the prosaposin gene may change the sulfatide binding specificity of the two encoded saposin B forms, to adapt to the variable sphingolipid composition of tissues (32). To test this hypothesis, lipids were extracted from brain, heart, and liver of $Pro^{\Delta 8/\Delta 8}$ and wild-type mice at different ages. No changes were detected in the levels of any of the lipids examined, including sulfatides, neutral glycosphingolipids, gangliosides, neutral phospholipids, neutral lipids, and free ceramides (Fig. 6), indicating that saposin B without the exon 8-encoded three-amino-acid insertion is active in degrading sulfatides in vivo.

DISCUSSION

Prosaposin has alternative splicing of a 9-bp exon 8 that results in different protein forms, with or without the exon 8-encoded amino acids (23, 44). Alternative splicing of prosaposin exon 8 is conserved in evolution, from zebra fish to humans. Moreover, exon 8-containing prosaposin RNA expression is conserved in mice and chickens during brain development (7).

Alternative splicing of the prosaposin gene was assumed as the mechanism responsible for differential sorting of the two prosaposin forms and for production of saposin B isoforms with different lipid binding specificities (32, 35). However, there was no conclusive evidence concerning the biological function of the exon 8-containing prosaposin (7, 16, 19, 32, 35). In the present work, gene-targeted technology was employed to create exon-specific knockout mice that are missing exon 8 of the mouse prosaposin gene. Strikingly, deletion of exon 8 did not lead to noticeable morphological changes and the $Pro^{\Delta 8/\Delta 8}$ mice are viable and fertile. Even the $Pro^{\Delta 8neo/\Delta 8neo}$

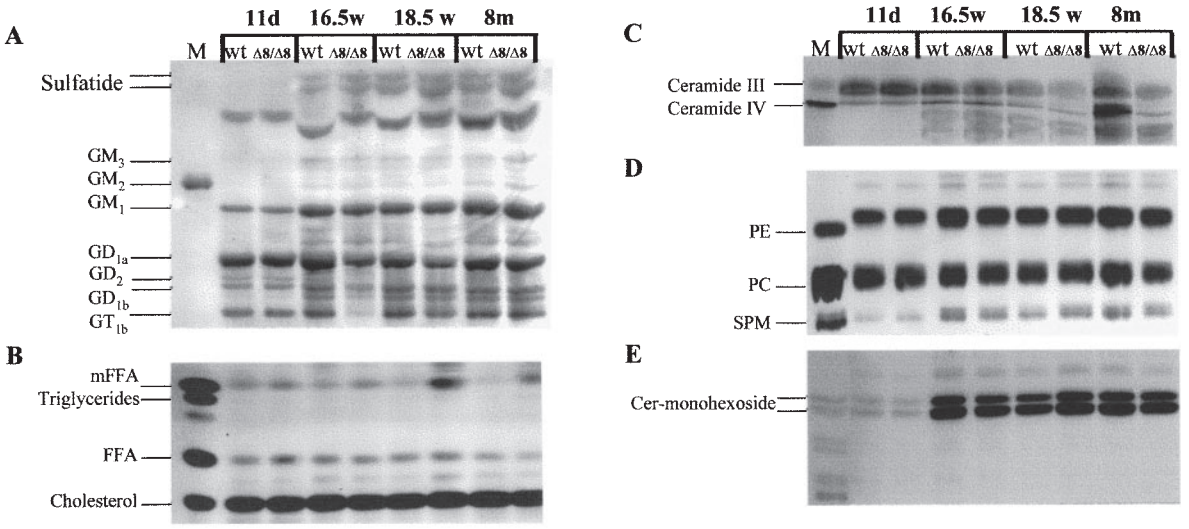

FIG. 6. Analysis of brain lipids from mice at different ages. Lipids were extracted from wild-type (wt) and $Pro^{\Delta 8/\Delta 8}$ (Δ8/Δ8) mouse brains at different ages with chloroform-methanol, as described in Materials and Methods. (A) Upper-phase lipids (gangliosides and sulfatides) were loaded on a Sephadex cartridge, and eluted solid-phase extract was spotted on TLC plates. The lower-phase lipids were loaded on an LC-NH_2 column, and solid-phase extraction fractions were separated by sequential elutions. (B) Neutral lipids. (C) Free ceramides. (D) Neutral phospholipids. (E) Neutral glycosphingolipids. PC, phosphatidylcholine; PE, phosphatidylethanolamine; SPM, sphingomyelin; FFA, free fatty acids; mFFA, methylated free fatty acids; d, days; w, weeks; m, months; M, marker.

mice, which have a ~50% reduction in the amount of prosaposin, do not present an obvious phenotype. $Pro^{\Delta 8/\Delta 8}$ mice showed no changes in lipid levels, suggesting that their metabolism is not altered and demonstrating that saposin B was functional.

The highest levels of the exon 8-containing prosaposin RNA were documented in brain, heart, and skeletal muscle (7, 19, 32, 68). The highest levels of unprocessed prosaposin were detected, by Western blot analysis, in brain, skeletal muscle, and heart, while the level of saposins was higher in visceral organs (53). Taken together, these observations strongly suggested that the exon 8-containing prosaposin RNA is translated to the unprocessed prosaposin form, which is more efficiently secreted, whereas prosaposin without exon 8 is translated to the prosaposin precursor of the four lysosomal saposins. However, mice lacking exon 8 ($Pro^{\Delta 8/\Delta 8}$) showed levels of secreted prosaposin in serum similar to those of wild-type mice. Since the $Pro^{\Delta 8neo/\Delta 8neo}$ mice did not show any change in litter size compared to wild type, we assume that prosaposin secretion in the reproductive system was normal as well. Therefore, while prosaposin expression is essential for normal mouse development and adult life (11), our results strongly suggest that the prosaposin splice variant containing exon 8 is dispensable for normal mouse development and adult life. It has already been documented that protein isoforms resulting from alternative splicing are dispensable. The proto-oncogene K-ras has two splice variants, K-ras4A and K-ras4B. K-ras4A-deficient mice, generated by gene targeting, were fertile and showed no histopathological abnormalities, demonstrating that K-ras4A is dispensable for normal mouse development, at least in the presence of functional K-ras4B (46).

Several studies demonstrated that prosaposin prevents nerve degeneration and promotes nerve regeneration in animal models of nerve injuries, including a strong protective action against ischemic and wound stab damage of hippocampal neurons (24, 30, 43, 54). The relative abundance of the exon 8-containing prosaposin isoform was recently shown to sharply decline following ischemia and stab wound in rat brain (19). The authors proposed that the accumulation of prosaposin lacking exon 8 following nerve injury may be due to a difference in transcription and/or turnover rate between the two alternatively spliced prosaposin mRNA species. Since we did not observe any change in prosaposin levels in the $Pro^{\Delta 8/\Delta 8}$ mice, it strongly suggests that there are no turnover rate or stability differences between the two mRNA species, at least under the tested conditions.

We have shown very recently that the expression level of exon 8-containing prosaposin mRNA reaches a peak after birth when synaptogenesis is extensive in mice (7). It is well documented that changes in synaptic transmission are associated with neurotoxicity after nerve injury (3, 25). Therefore, it is possible that the three-amino-acid-containing prosaposin has a role, as a ligand, in synaptogenesis and/or synaptic transmission.

In summary, our results demonstrate that mice lacking exon 8 of prosaposin are healthy and fertile. They also have unchanged lipid metabolism. These data indicate that saposin B without the exon 8-encoded three-amino-acid insertion is active in degrading sulfatides in vivo. We also demonstrated that the prosaposin form without the extra three amino acids is also secreted in vivo and in tissue culture cells. It is possible that both prosaposin forms have extracellular functions. Our results strongly suggest that the prosaposin variant containing the exon 8-encoded three amino acids is dispensable for normal mouse development and fertility as well as for prosaposin secretion and its lysosomal function, at least in the presence of the prosaposin variant lacking the exon 8-encoded three amino acids. However, we cannot exclude the possibility that there are subtle changes between the normal and the mutant

($Pro^{\Delta 8/\Delta 8}$ and $Pro^{\Delta 8neo/\Delta 8neo}$) mice that have not been encountered yet.

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